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# **BOTULINUM NEUROTOXIN AND TETANUS TOXIN**

**EDITED BY**

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# Bacterial Sources of Clostridial Neurotoxins

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## I. Introduction

The organisms capable of producing botulinum and tetanus neurotoxins belong to the genus *Clostridium*. Clostridia are anaerobic, spore-forming rods that are considered gram-positive, although some species do not actually show the positive Gram stain reaction. Some are gram-positive in young cultures but gram-negative in older cultures, while others show a mixture of positively and negatively stained cells. The most striking morphological feature is the spore, which since it is usually wider in diameter than the cell itself, causes the cell to swell and gives rise to the spindle shape to which the name *Clostridium* refers (*closter*, a Greek word meaning *spindle*).

Anaerobic organisms, according to one definition, "(i) generate energy and synthesize their substance without recourse to molecular oxygen; and (ii) demonstrate a singular degree of adverse oxygen-sensitivity which renders them unable to grow under an atmosphere of air" (Morris, 1975). Some species of clostridia are aerotolerant; they will grow on agar surfaces in the presence of air (Smith and Williams, 1984). They can be distinguished from members of the genus *Bacillus* by their lack of catalase. Clostridia lack cytochrome oxidase, which is involved in aerobic metabolism, and also lack the enzymes catalase and peroxidase, which protect biological systems against the deleterious effects of oxygen and oxygen derivatives (Decker *et al.*, 1970; Morris, 1975). Superoxide dismutase may be present in some obligate anaerobes, including clostridia (Hewitt and Morris, 1975). The most important nutrients used by anaerobes are monosaccharides, amino acids, unsaturated acids, keto acids, and alcohols, which are used in hydrogenation and dehydrogenation reactions yielding energy conserved by substrate level phosphorylations. Saturated fatty acids cannot be used to produce energy in the absence of oxygen for thermodynamic and mechanistic reasons (Decker *et al.*, 1970). These compounds are generally found as metabolic end products.

Clostridia are found in soils; sediments in lakes, rivers, and marine waters; intestinal tracts of humans and animals; decaying animal carcasses and other organic matter; and in sewage, where there is an abundance of energy sources and an anaerobic environment. They are thus widely distributed in nature and can readily find their way into wounds. If a low oxidation-reduction potential in the tissue results from disruption of the circulation and the activity of facultative organisms, the clostridia can grow in the wound.

Currently, 83 species of *Clostridium* are listed in Bergey's Manual (Cato *et al.*, 1986). Fourteen species are considered pathogenic (Smith and Williams, 1984). Most of the pathogenic species are well noted for their ability to produce toxins that play a role in the pathology of various infections involving clostridia. Examples are local tissue destruction in gas gangrene, necrotic enteritis caused by *Clostridium perfringens* type C, and paralytic effects in tetanus and wound botulism.

## II. Discovery

The recognition of neurotoxic diseases tetanus and botulism caused by an infectious agent showed that tetanus could be transmitted to a rabbit by inoculating an infected wound, while at the same time, tetanus in these animals (Koch, 1876). The clinical manifestations of tetanus in these organisms in soil that contain the organism and its spores. The organism was first isolated (Bytchenko, 1981). Pure culture of tetanus when injected into animals could be immunized and that the animals' serum (Miles, 1975).

Botulism, or sausage poisoning, was first described in the eighteenth century, but it was not until the late nineteenth century and its toxicopathic nature involving three fatal, ten cases in Eliezelles, Belgium. The first case was in a young man, Ermengem, and a thorough investigation years later (van Ermengem, 1897) showed that the uncooked ham. Portions of the ham caused signs of illness in monkeys and rats. Injections of filtrates of mace caused symptoms and death in rats. van Ermengem suspected that the filtrate was in tetanus and diphtheria. He found in cultures of the ham as well as in the victims of the outbreak. Cultures of the same effect on susceptible animals. The organism was named *Bacillus botulinus*.

While the bacteriology of botulism has advanced, the experience with botulism has revealed a variety of toxin types and a diversity of clinical manifestations. Furthermore, it was eventually discovered that the organism is capable of growth in the

on

and tetanus neurotoxins belong to a group of spore-forming rods that are characterized by the fact that they do not actually show the characteristic positive reaction in young cultures but show a mixture of positively and negatively reacting cells. One of the distinguishing morphological features is the spore, which is located at one end of the cell itself, causes the cell to swell, and the name *Clostridium* refers to this feature.

In definition, "(i) generate energy from molecular oxygen; and (ii) lack the sensitivity which renders them obligate anaerobes" (Morris, 1975). Some species of clostridia grow on surfaces in the presence of air, but are distinguished from members of the aerobic bacteria by the fact that they lack cytochrome oxidase, lack the enzymes catalase and peroxidase, and are resistant to the deleterious effects of oxygen (Morris, 1975). Superoxide dismutase, an enzyme found in aerobes, including clostridia, is used by anaerobes as a defense against oxygen. Fermentation reactions yielding energy from carbohydrates cannot be coupled to thermodynamic and mechanical work. Wounds are generally found as

in soil, rivers, and marine waters; in animal carcasses and other sources of abundance of energy sources and are distributed in nature and can be characterized by a low redox potential in the environment and the activity of facultative

as found in Bergey's Manual (Cato et al., 1975). Clostridia are pathogenic (Smith and Williams, 1975) and are known for their ability to produce infections involving clostridia. Clostridia cause necrotic enteritis caused by the toxin and effects in tetanus and wound

## II. Discovery of Neurotoxic Clostridia

The recognition of neurotoxic bacteria occurred during investigation of the diseases tetanus and botulism. In 1867, Pirogov hypothesized that tetanus was caused by an infectious agent (Bytchenko, 1981). Seventeen years later, Rattone showed that tetanus could be transmitted from a human suffering from the disease to a rabbit by inoculating the rabbit with material from the patient's infected wound, while at the same time Nicolaier discovered that implantation of soil samples into mice, rabbits, and guinea pigs could cause symptoms of tetanus in these animals (Kobel and Marti, 1985). Nicolaier postulated that the clinical manifestations of tetanus were due to a poison produced in patients by organisms in soil that contaminated their wounds. The "drumstick" form of the organism and its spores was described by Rosenbach and Flugge, and the organism was first isolated in pure culture by Kitasato from a patient in 1889 (Bytchenko, 1981). Pure cultures and cell-free filtrates were shown to cause tetanus when injected into animals. Behring and Kitasato demonstrated that animals could be immunized with tetanus toxin modified by iodine trichloride and that the animals' serum contained neutralizing antibodies (Wilson and Miles, 1975).

Botulism, or sausage poisoning, had been documented since the end of the eighteenth century, but it was not until 100 years later that its bacterial etiology and its toxicopathic nature came to light. In 1895, an outbreak of botulism involving three fatal, ten very serious, and several milder cases occurred in Ellezelles, Belgium. The incident was investigated by Emile Pierre Marie van Ermengem, and a thorough report of his extensive study was published about 3 years later (van Ermengem, 1897). The outbreak was caused by a salt-cured, uncooked ham. Portions of macerated ham fed to mice, guinea pigs, and monkeys caused signs of illness indicative of botulism and subsequently resulted in death; feedings to rats and cats had little effect, but subcutaneous injection caused symptoms and death. Dogs and chickens were unaffected by feeding or injections. Filtrates of macerated ham had the same effect as the macerate itself. van Ermengem suspected that a bacterial agent was responsible for the toxin, as in tetanus and diphtheria. He consistently found an anaerobic sporulating bacillus in cultures of the ham as well as in a culture of spleen from one of the deceased victims of the outbreak. Cultures and culture filtrates of those organisms had the same effect on susceptible animals as did the ham macerates. The organism was named *Bacillus botulinus*.

While the bacteriology of tetanus has remained relatively simple, the experience with botulism has revealed a rather complicated picture, for a multiplicity of toxin types and a diversity of organisms capable of producing the botulinum toxins were discovered during the 90 years following van Ermengem's studies. Furthermore, it was eventually established that botulism could occur as a result of growth of the organism in wounds (Merson and Dowell, 1973) and because of

colonization of the intestine in infants (Midura and Arnon, 1976, Pickett *et al.*, 1976) and others (McCroskey and Hatheway, 1988).

Although the etiologic agent of tetanus was discovered first, it will be easier to make the presentation of its biologic features after first presenting the complexities of the group of organisms capable of producing botulinum toxins. This order of presentation will also be more consistent with the title of this book.

### III. Organisms That Produce Botulinum Neurotoxin

#### A. Toxin Types

Thirteen years after the report of van Ermengem (1897), Leuchs (1910) reported that the toxin from an organism responsible for a subsequent outbreak of botulism (Landman, 1904) could be distinguished from that of van Ermengem's bacillus on the basis of differential neutralization with specific antitoxins. In 1919, type A and B toxin designations were established using strains isolated from outbreaks of human botulism in the United States (Burke, 1919). The van Ermengem and Landman strains were no longer available by that time, but it is probable that the former would correspond to type B and the latter to type A. The generic name *Clostridium* was adopted in place of *Bacillus* as recommended by the Committee on Classification of the Society of American Bacteriologists; *Clostridium* was chosen to include anaerobic spore-forming organisms, while *Bacillus* included only aerobic organisms (Bengtson, 1924). The species *Clostridium botulinum* was expanded to include a diversity of culturally distinct organisms that produce botulinum neurotoxin discovered over the next several decades (Smith and Williams, 1984).

Organisms that caused botulism in chickens in the United States (Bengtson, 1922) and in cattle in Australia (Seddon, 1922) were of a new type, designated type C; toxin from both strains was neutralized with the Bengtson strain antitoxin, while antitoxin to the Seddon strain neutralized only the homologous toxin. The two subtypes of type C toxin were designated C<sub>α</sub> and C<sub>β</sub> respectively (Gunnison and Meyer, 1929). An organism similar to the type C strains but producing yet another type of toxin caused an outbreak of botulism in cattle in South Africa; this organism was designated toxin type D (Meyer and Gunnison, 1928). Two human botulism outbreaks in 1934 resulting from contaminated fish in New York State (Hazen, 1937) and in the Ukraine (Kushnir *et al.*, 1937) were due to type E botulinum toxin (Gunnison *et al.*, 1936–1937). Type F botulinum toxin was recognized in 1958 in the investigation of a botulism outbreak in Denmark due to liver paste (Moller and Scheibel, 1960). Thus, botulinum toxins of type A through F and the organisms that produce them were discovered through the investigation of botulism outbreaks in humans (types A, B, E, and F) and animals (types C and D).

Type G botulinum toxin was encountered in low concentration in a soil survey culture by Gimenez and Ciccarelli (1970b). The toxigenic organism was

#### 1. Bacterial Sources of Clostric

recovered from the culture activity, which ordinarily p Sonnabend *et al.* (1981) req specimens, but thus far ther ulism has occurred in hur conditions.

Serologic analysis of multiple components are pr and a small amount of D tox minor amount of C<sub>1</sub>. Type C a binary toxin consisting of respectively) that are not c hypotension, hemorrhaging lungs and in the trachea (Sir to be due to changes in me

Variation in serologic same toxin type has been noi groups of strains of type A to of antitoxin in the neutraliz: ment for neutralization. A si noted in the study of an isc (1981). A strain isolated from 93% type A and 7% type F produce about 90% type B: with botulism (Hatheway a

Thus, seven serologic and minor variations and co of botulinum toxins is rather single serologic type.

#### B. Physiology and Production

It was noted early that str variable cultural characteri strain grew well at 18–25°C grew better at the higher ter tion between the two strain: strains was confusing, and systematic comparisons w teolytic activities of Americ type B strain, and found th meat, egg, and serum pro: three types of protein. The

d Arnon, 1976, Pickett *et al.*, 1978).

is discovered first, it will be a major contribution after first presenting the facts about the production of botulinum toxins. The content is consistent with the title of this book.

## Produce toxin

(1897), Leuchs (1910) reported a subsequent outbreak of botulism from that of van Ermengem's with specific antitoxins. In 1919, published using strains isolated from states (Burke, 1919). The van was available by that time, but it is type B and the latter to type A. The *Bacillus* as recommended by the American Bacteriologists; re-forming organisms, while (Bengtson, 1924). The species diversity of culturally distinct covered over the next several

in the United States (Bengtson, 1924), were of a new type, designated as the Bengtson strain antitoxin, by the homologous toxin. The type C<sub>β</sub> respectively (Gunnison, 1928). Type C strains but producing yet in cattle in South Africa; this (Gunnison, 1928). Two human fatalities in New York State (Leuchs, 1937) were due to type E and type F botulinum toxin was recovered in Denmark due to liver and muscle toxins of type A through F and through the investigation of humans and animals (types C and D). In a low concentration in a soil. The toxigenic organism was

recovered from the culture in spite of its low toxigenicity and lack of lipase activity, which ordinarily provides a recognizable marker for *C. botulinum*. Sonnabend *et al.* (1981) reported isolating *C. botulinum* type G from autopsy specimens, but thus far there has not been adequate evidence that type G botulism has occurred in humans, nor in animals except under experimental conditions.

Serologic analysis of the toxins of type C and D organisms revealed that multiple components are produced by these strains. Type C<sub>α</sub> produces C<sub>1</sub>, C<sub>2</sub>, and a small amount of D toxin, while type D produces a major amount of D and a minor amount of C<sub>1</sub>. Type C<sub>β</sub> produces only C<sub>2</sub> toxin (Jansen, 1987). C<sub>2</sub> toxin is a binary toxin consisting of two separate molecules (55,000 and 105,000 Da, respectively) that are not covalently linked. It is not a neurotoxin but causes hypotension, hemorrhaging in the lungs, and a collection of fluid around the lungs and in the trachea (Simpson, 1982). The *in vivo* effects of C<sub>2</sub> toxin appear to be due to changes in membrane permeability or cellular secretion.

Variation in serologic characteristics of toxins from different strains of the same toxin type has been noted. Ciccarelli and Gimenez (1971) differentiated two groups of strains of type A toxin producers, one with toxin consuming a low level of antitoxin in the neutralization test and a second with a high antitoxin requirement for neutralization. A similar difference in toxins from type B organisms was noted in the study of an isolate from an infant with botulism (Hatheway *et al.*, 1981). A strain isolated from soil produced two identifiable types of toxin, about 93% type A and 7% type F (Gimenez and Ciccarelli, 1970a). Two strains that produce about 90% type B and 10% type F toxin have been isolated from infants with botulism (Hatheway and McCroskey, 1987).

Thus, seven serologic types of botulinum neurotoxin have been identified, and minor variations and combinations have been noted. The serologic spectrum of botulinum toxins is rather complex compared with tetanus toxin, which has a single serologic type.

## B. Physiologic Differences among Organisms Producing Botulinum Neurotoxin

It was noted early that strains of organisms that produce botulinum toxin have variable cultural characteristics. Leuchs (1910) noted that the van Ermengem strain grew well at 18–25°C but poorly at 35–37°C, while the Landman bacillus grew better at the higher temperatures. Differences were noted in sugar fermentation between the two strains. Information on milk protein digestion for these two strains was confusing, and the strains were no longer available for study when systematic comparisons were being made. Bengtson (1924) compared proteolytic activities of American type A and type B strains with those of a European type B strain, and found the American strains of both types were proteolytic for meat, egg, and serum proteins while the European strain was negative for all three types of protein. The latter strain was judged to conform most closely to



van Ermengem's description of his original isolate. In Bengtson's study (1924), all seven type C strains were nonproteolytic. Characteristics of organisms that produce type D toxin coincide with those of type C organisms.

Organisms that produce type E toxin resemble the nonproteolytic European type B organisms in their lower temperature optimum, in the lower heat resistance of the spores, and in their lack of proteolytic activity. Although very few type F botulism cases have been recorded, environmental studies have yielded strains approximately equally divided between proteolytic and nonproteolytic groups. The discovery of the organism that produces type G toxin expanded the nomenclature dilemma by adding another organism with even more disparate characteristics than encountered with the previous types and groups: the type G organism is asaccharolytic, and negative for lipase which generally serves as a key characteristic for isolating botulinum toxin-producing organisms (Gimenez and Ciccarelli, 1970a). However, the name *Clostridium botulinum* was applied to this as well as to all other organisms capable of producing botulinum neurotoxin.

At this point, four physiologic groups of *C. botulinum* are recognized (Smith, 1977). Each is designated with a Roman numeral (I, II, III, IV; Table I). Group I organisms are usually referred to as "proteolytic," while Group II organisms are "saccharolytic" (Cato *et al.*, 1986). Type B and type F toxins can be produced by strains belonging to either Group I or Group II. Group III contains only organisms that produce type C or type D toxin. They may or may not digest complex proteins, but they are distinguished from both Groups I and II by production of significant amounts of propionic acid. Group IV consists of organisms that produce type G neurotoxin. Recently, classification of neurotoxic organisms has become even more complicated since strains of clostridial species, *Clostridium baratii* and *Clostridium butyricum*, that also produce botulinum neurotoxin have been isolated (Hall *et al.*, 1985; McCroskey *et al.*, 1986).

### C. Related Nontoxigenic Organisms

Nontoxigenic clostridial species phenotypically related to Groups I, III, and IV are *Clostridium sporogenes*, *Clostridium novyi*, and *Clostridium subterminale*, respectively (Table I). Nontoxigenic organisms similar to Group II are encountered but have not been given a species name. The existence of these nontoxigenic organisms has engendered speculation that each group could be considered a separate species consisting of toxigenic and nontoxigenic strains.

### D. Genetic Studies

The genetic relatedness of toxigenic strains of *C. botulinum* Groups I, II, and III and their nontoxigenic counterparts has been studied (Lee and Riemann, 1970a, 1970b; Nakamura *et al.*, 1977, 1983; Wu *et al.*, 1972). Suen (1986) recently completed a DNA hybridization study on *C. botulinum* type G (Group IV) and strains identified as *Clostridium subterminale* and *Clostridium hastiforme*. In

Table I Differential Characteristics for the Four Physiologic Groups of *Clostridium botulinum*

e. In Bengtson's study (1924), characteristics of organisms that are C organisms.

able the nonproteolytic European strain, in the lower heat resistance ability. Although very few type F studies have yielded strains proteolytic and nonproteolytic groups. Type G toxin expanded the nomenclature even more disparate characteristics and groups: the type G organism generally serves as a key characteristic for organisms (Gimenez and Cicciocioppo) and was applied to this as being botulinum neurotoxin.

*C. botulinum* are recognized by numeral (I, II, III, IV; Table I). 'proteolytic,' while Group II is not. Type B and type F toxins can occur in Group I or Group II. Group III produces type D toxin. They may or may not be isolated from both Groups I and II. Group IV produces lactic acid. Group IV consists of only one strain, classification of neurotoxins is complicated since strains of clostridial species, that also produce botulinum toxin, have been reported (McCroskey *et al.*, 1985; McCroskey *et al.*,

## Organisms

related to Groups I, III, and IV and *Clostridium subterminale*, similar to Group II are encountered. The existence of these nontoxigenic strains could be considered nontoxigenic strains.

## ies

*botulinum* Groups I, II, and III are isolated (Lee and Riemann, 1970a, 1972). Suen (1986) recently isolated *botulinum* type G (Group IV) and *Clostridium hastiforme*. In

Table I Differential Characteristics for the Four Physiologic Groups of *Clostridium botulinum*

Group	Type of Toxin	Milk Digestion	Glucose Fermentation	Lipase	Metabolic Products <sup>a</sup>		Phenotypically Related <i>Clostridium</i>
					Volatile	Nonvolatile	
I	A,B,F	+	+	+	A,iB,B,iV	PP	<i>C. sporogenes</i>
II	B,E,F	-	+	+	A,B		<i>C. novyi</i>
III	C,D	±	+	+	A,P,B	PA	<i>C. subterminale</i>
IV	G	+	-	-	A,iB,B,iV		<i>C. subterminale</i>

<sup>a</sup>Metabolic acid end products in peptone-yeast extract-glucose medium. A, acetic; P, propionic; iB, isobutyric; B, butyric; iV, isovaleric; PP, phenylpropionic; PA, phenylacetic.

general, all strains within Group I and Group II, regardless of toxin type, are closely related, while strains of the same or different toxin types belonging to different physiologic groups have low relatedness. Phenotypically similar nontoxigenic organisms may show high, intermediate, or low genetic relatedness to their toxigenic counterparts. It appears there is heterogeneity among strains identifiable as *C. sporogenes*. Some are closely related to Group I *C. botulinum* while others are not (Lee and Riemann, 1970a; Nakamura *et al.*, 1977; Wu *et al.*, 1972). The type strain of *C. sporogenes* is apparently among the latter (Suen *et al.*, 1986).

There appears to be some genetic diversity among strains in Group III (Lee and Riemann, 1970a; Nakamura *et al.*, 1983). At least two genetic groups of toxigenic organisms are intermediately related to each other as well as to *C. novyi* types A and B, and *C. haemolyticum*. Suen (1986) found that nine strains of *C. botulinum* type G were all closely related to each other as well as to two strains identified as *C. subterminale* and one of *C. hastiforme*; they were unrelated to the type strains of those two species, however.

In summary, the four groups of *C. botulinum* are clearly distinguishable from each other genetically; within each physiologic group there may be different degrees of relatedness. In each group, phenotypically similar, nontoxigenic organisms exist, some of which may be closely related to some of the toxigenic organisms in that group.

### E. Bacteriophages

Bacteriophages associated with Groups I, II, and III *C. botulinum* have been documented (Inoue and Iida, 1968; Eklund *et al.*, 1969; Dolman and Chang, 1972). Only with Group III strains has there been an association between phages and toxigenicity of the bacteria (see Chapter 2). Type C and D strains cured of their phages no longer produce toxin. Such nontoxigenic organisms could be converted to toxigenic forms, producing either type C or type D toxin, by reinfecting the organism with the phage isolated from the corresponding toxigenic strain (Eklund and Poysky, 1974). Subsequently it was demonstrated that a type C strain cured of its phage could be converted to an organism that was indistinguishable from *C. novyi* type A by infecting it with a phage (NA1) derived from a strain of *C. novyi* (Eklund *et al.*, 1974). Phage studies suggest that *C. botulinum* type C<sub>β</sub> is derived from C<sub>α</sub> after C<sub>α</sub> loses the phage responsible for producing type C<sub>1</sub> toxin (Jansen, 1987).

### F. Plasmids

Plasmid studies of *C. botulinum* have been pursued because of the interest in the genetics of toxigenicity in these organisms. Strom *et al.* (1984) isolated plasmids from all four groups of *C. botulinum*, including strains of all toxin types. Multiple plasmids were found in strains of Group I, types A and B, and Group III,

### I. Bacterial Sources of Clostridi

types C and D. Single plasmid Group II strains of types B and Group I type F (11.5 MDa, five) and type G (73 MDa, six) strains the plasmids in that study. *botulinum* type A with different organisms that initially lacked had been eliminated after growth. Toxigenicity was lost by some; there was no correlation with plasmid.

### G. Cellul

The organisms in all four groups were 1.6 to 22.0 μm wide and 1.6 to 22.0 μm long.

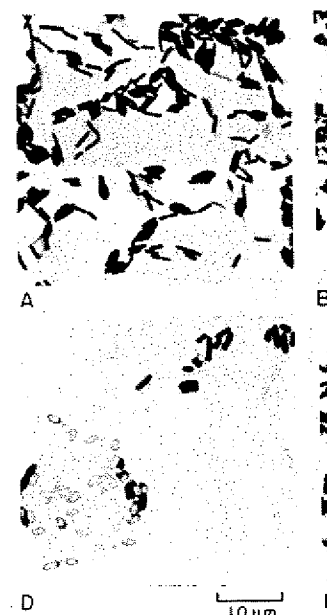


Fig. 1. Photomicrographs of *Clostridium botulinum* and *Clostridium tetani* indicated by bar in lower left: (B) *Clostridium botulinum* type B, Group II (n type E (CDC5545)). (E) *Clostridium tetani* (CDC6202). Photograph

II, regardless of toxin type, are different toxin types belonging to the same species. Phenotypically similar nontoxic, or low genetic relatedness to the toxin type is heterogeneity among strains related to Group I *C. botulinum* (Suen et al., 1977; Nakamura et al., 1977; Wu et al., 1977) apparently among the latter (Suen et al., 1977).

among strains in Group III (Lee et al., 1986). At least two genetic groups of Group III exist to each other as well as to *C. botulinum*. Lee et al. (1986) found that nine strains of Group III to each other as well as to two strains of *C. botulinum* were unrelated to each other.

*C. botulinum* are clearly distinguishable from other clostridia. In this logic group there may be different toxin types, phenotypically similar, nontoxicogenic strains related to some of the toxigenic strains.

## Genetics

Group III *C. botulinum* have been studied (Lee et al., 1986; Dolman and Chang, 1986) and an association between phages and Group III has been found.

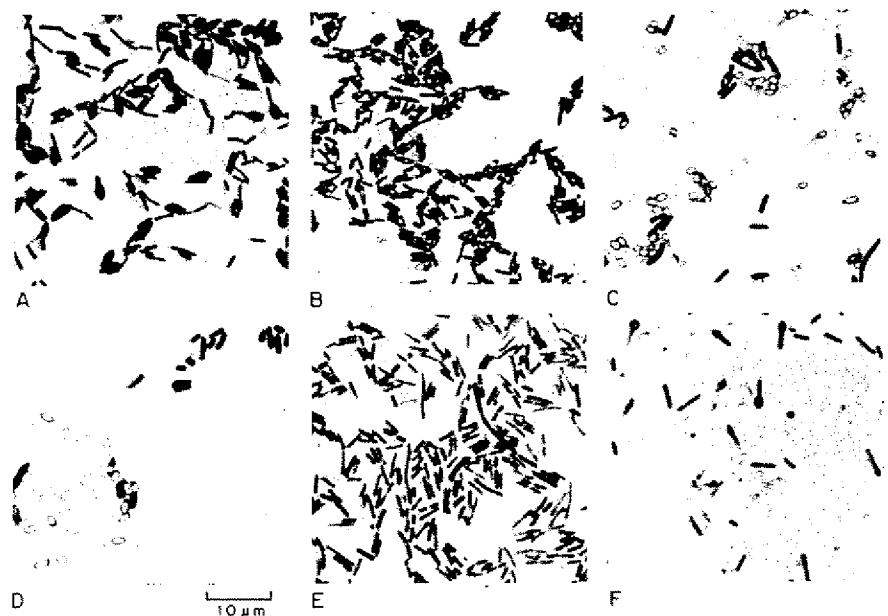
Type C and D strains cured of toxin genes could be converted to type C or type D toxin, by infection with the corresponding toxin gene. Recently it was demonstrated that a nontoxicogenic organism that was converted to an organism that was toxigenic by infecting it with a phage (NA1) (Lee et al., 1974). Phage studies suggest that *C. botulinum* loses the phage responsi-

ble because of the interest in the toxin gene. Lee et al. (1984) isolated plasmids from strains of all toxin types. Multitoxigenic strains of types A and B, and Group III,

types C and D. Single plasmids that differed between strains were found with Group II strains of types B and E. A single, uniform plasmid was found for Group I type F (11.5 MDa, five strains), Group II type F (2.2 MDa, four strains), and type G (73 MDa, six strains). No phenotypic functions were determined for the plasmids in that study. Weickert et al. (1986) studied 12 strains of *C. botulinum* type A with differing plasmid profiles and observed toxigenicity in organisms that initially lacked plasmids as well as in organisms whose plasmids had been eliminated after growth in medium containing sodium deoxycholate. Toxigenicity was lost by some of the isolates obtained after treatment, but there was no correlation with plasmid loss.

## G. Cellular Morphology and Spores

The organisms in all four groups are medium to large rods, measuring 0.5 to 2.0  $\mu\text{m}$  wide and 1.6 to 22.0  $\mu\text{m}$  long (Cato et al., 1986). They appear singly or in



**Fig. 1.** Photomicrographs of gram-stained smears of egg yolk agar cultures of *Clostridium botulinum* and *Clostridium tetani*. Uniform magnification for all strains indicated by bar in lower left figure. (A) *Clostridium botulinum* type A (CDC5356). (B) *Clostridium botulinum* type B, Group I (proteolytic) (CDC5448). (C) *Clostridium botulinum* type B, Group II (nonproteolytic) (CDC5569). (D) *Clostridium botulinum* type E (CDC5545). (E) *Clostridium botulinum* type G (CDC1353). (F) *Clostridium tetani* (CDC6202). Photography by Don Howard.

pairs and are variable in their Gram-stain reactions. Spores are subterminal and are larger in diameter than the cell, thus causing swelling of the rod in the area of the spore. Photomicrographs of some representative strains are shown in Fig. 1 and 2. Available strains of *C. botulinum* type G do not sporulate readily, and usually no spores are seen on smears of those cultures. However, Lynt *et al.* (1984) succeeded in producing spore crops from two strains for heat resistance studies.

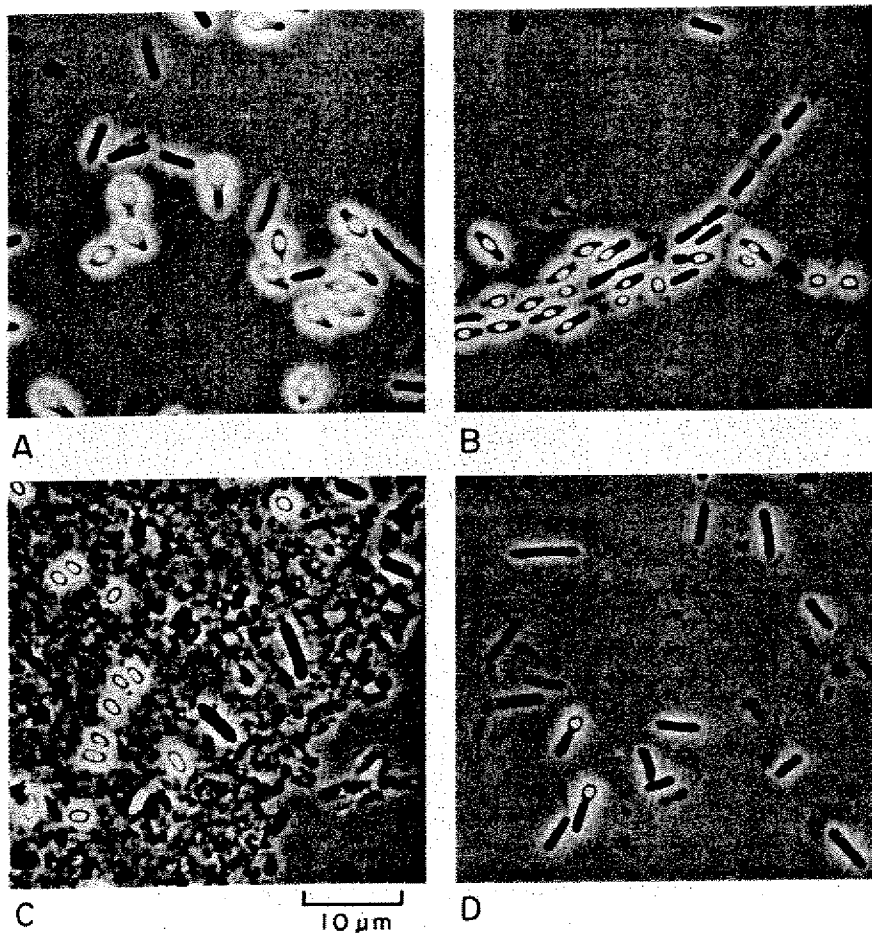


Fig. 2. Photomicrographs of phase-contrast views of wet-mount suspensions of egg yolk agar cultures of *Clostridium botulinum* and *Clostridium tetani*. Uniform magnifications for all strains indicated by bar in lower left picture. (A) *Clostridium botulinum* type A (CDC5356). (B) *Clostridium botulinum* type B, Group I (proteolytic) (CDC5448). (C) *Clostridium botulinum* type E (CDC5545). (D) *Clostridium tetani* (CDC6203). Photography by Don Howard.

## I. Bacterial Sources of Clostridia

When spores are not shown by survival after heat treatment of organisms. Group I spores are treatment in a boiling waterbath survive 80°C for 10 min. All of Group II organisms. Growth between those of Groups I and populations of spores: 99% of Group II; the remainder show spores (Lynt *et al.*, 1984).

## H. Cultural Characteristics

All four groups of organisms grow on the surface of agar medium in a discrete colonies on agar surface. The primary differentiating characteristics are shown in Table 1. Groups I, III, and IV is appropriate. Group II organisms have a little or no growth at 45°C; as been reported (Eklund *et al.*

## I. Other Bacteria

Toxigenic organisms responsible for clostridial species unrelated to those isolated from an infant with botulism (Hall *et al.*, 1985). Organisms isolated in Italy were identical to *C. butyricum* (A frequent DNA hybridization study related to the type strains of New Mexico isolate was 86% and 3.5% divergence (unpublished) from the first Italian type 19398, with 1% divergence

## J. Food

*C. botulinum* is found in various lakes and seas in most parts of the United States have shown either type A or B (most likely

is. Spores are subterminal and swelling of the rod in the area of the spore is shown in Fig. 1. Some strains do not sporulate readily, and cultures. However, Lynt *et al.* selected two strains for heat resistance

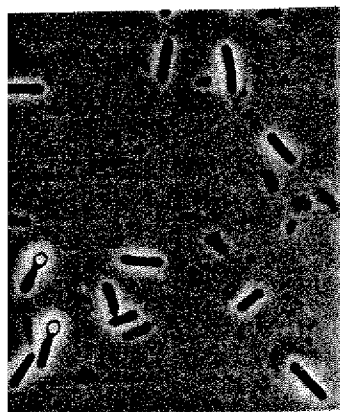
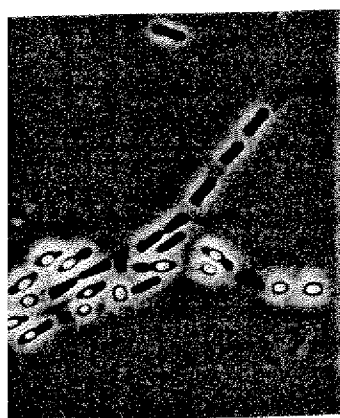


Fig. 1. Wet-mount suspensions of egg yolk medium. (A) *Clostridium botulinum* type B, Group I (prototype CDC5545). (D) *Clostridium tetani*. Uniform magnification.

When spores are not visible on stained smears, their presence may be shown by survival after heat or alcohol treatments designed to destroy vegetative organisms. Group I spores are heat resistant; many strains maintain viability after treatment in a boiling waterbath for 2 hr. Group II spores are not, often failing to survive 80°C for 10 min. Alcohol treatment is recommended for selecting spores of Group II organisms. Group III spores have a heat resistance intermediate between those of Groups I and II. *C. botulinum* type G appears to produce two populations of spores: 99% or more have a heat liability similar to spores of Group II; the remainder show heat resistance comparable to that of Group I spores (Lynt *et al.*, 1984).

## H. Cultural and Physiologic Characteristics

All four groups of organisms are strict anaerobes; they will not grow on the surface of agar medium in ambient atmosphere or in a candle jar. They form discrete colonies on agar surfaces, and the lipase reaction, detectable on egg yolk medium, is the primary differential characteristic of all except type G. Key characteristics are shown in Table I. The optimum growth temperature range for Groups I, III, and IV is approximately 30° to 37°C, with good growth at 45°C. Group II organisms have a lower optimum temperature range, 25° to 30°C, with little or no growth at 45°C; a minimum growth temperature as low as 3.3°C has been reported (Eklund *et al.*, 1967).

## I. Other Clostridia That Produce Botulinum Neurotoxin

Toxigenic organisms responsible for three cases of infant botulism resembled clostridial species unrelated to the four groups of *C. botulinum*. The organism isolated from an infant with type F botulism in New Mexico resembled *C. baratii* (Hall *et al.*, 1985). Organisms responsible for two cases of type E infant botulism in Italy were identical in all phenotypic characteristics except their toxigenicity to *C. butyricum* (Aureli *et al.*, 1986; McCroskey *et al.*, 1986). Subsequent DNA hybridization studies showed that the toxigenic isolates were closely related to the type strains of the species they resembled (Suen *et al.*, 1988). The New Mexico isolate was 86% related to *C. baratii* ATCC 27638, with no more than 3.5% divergence (unpaired nucleotides within related sequences). The isolate from the first Italian type E case was 78% related to *C. butyricum* ATCC 19398, with 1% divergence.

## J. Habitat and Distribution

*C. botulinum* is found in various frequencies in soils as well as in sediments in lakes and seas in most parts of the world (Hauschild, 1989). Soil surveys in the United States have shown widespread distribution, with most isolates being either type A or B (most likely all Group I), with the former predominating in the

western half of the country and the latter in the east (Smith, 1978). The organisms are generally not found in normal human feces (Easton and Meyer, 1924; Dowell *et al.*, 1977b). They are occasionally found in feces of normal farm animals but do not appear to be a significant part of the normal flora (Easton and Meyer, 1924). Type C organisms are often present in carrion and thus are responsible for epizootics. Poultry by-products used as feed, bedding, and grassland fertilizer have been implicated as sources of outbreaks in cattle (Egyed, 1987; Smart *et al.*, 1987).

### K. Toxin Detection and Identification

The most reliable means of detecting and identifying botulinum toxin in cultures and extracts from unknown specimens is by using mouse toxicity and neutralization tests (Hatheway, 1988). Samples (0.4 ml) of culture supernatants or filtrates, or extracts of specimens, are injected intraperitoneally into mice (approximately 20 g) with and without monospecific and polyvalent botulinum antitoxins. Mice injected with unneutralized neurotoxin die within 6–48 hr, depending on dose. Signs of botulism in the mice can be seen before death. The toxin type is established by observing which specific antitoxin neutralizes the toxicity. *In vitro* toxin assays may be more applicable when assaying numerous samples of toxin from a known source, for example, samples of fractions in a chromatographic purification process. The various procedures for assaying the toxin and merits of these various procedures are discussed more fully in Chapter 14.

### L. Isolation of Organisms

Recovery of *C. botulinum* for clinical specimens and food and soil samples is relatively easy because of the possibility of eliminating much of the competing flora by spore selection and because of the differential characteristic lipase. Selective egg yolk agar media can also be helpful (Dezfulian *et al.*, 1981; Mills *et al.*, 1985). Heat treatment is effective for recovering heat-resistant spores. To recover spores of Group II organisms, either a lower temperature for heat treatment or alcohol treatment (Johnston *et al.*, 1962) is sometimes necessary. Occasionally it is difficult to isolate toxigenic organisms because nontoxigenic, lipase-positive organisms such as *C. sporogenes* are also present in high numbers. An antitoxin overlay immunodiffusion method has been proposed to distinguish between colonies of toxigenic and nontoxigenic organisms on agar plates (Ferreira *et al.*, 1983). Initial cultures are inoculated into chopped meat or chopped meat–glucose–starch medium (Dowell *et al.*, 1977a); organisms are isolated by streaking enrichment cultures or suspensions of the original specimen on selective or nonselective egg yolk agar (Hatheway, 1988). Single colonies are picked from the agar surface and inoculated into chopped meat–glucose–starch medium. Toxigenicity is established by mouse toxicity and neutralization tests on culture supernatants. Enrichment cultures should also be tested for toxin,

### I. Bacterial Sources of Clostridi

especially since lipase-negative *baratii*, and *C. butyricum* can be fully characterized using methods (Holdeman *et al.* (1977), and

## IV. Org Te

### A. Toxins P

The neurotoxin produced by strains of *C. tetani* that are phenotypically similar. Base types have been recognized (1984).

Physiologic characteristics of strains of *C. tetani* that are their toxigenicity (Fildes, 19 on blood agar plates. It is do of tetanus (Adams *et al.*, 19

### B. Cellul

*C. tetani* is usually a gram-staining in portions near the s cultures (less than 18 hr old) usually appear gram-negative. Most strains are motile by r Spores are usually in a termir Photomicrographs of represe generally survive moderate stroyed after heating at 100°C so that heating at 100°C for ensure sterility (Adams *et a phenol, and even formaldeh turaldehyde, and hydrogen j agents.*

### C. Cultural :

*C. tetani* is a strict anaerobe blood agar, peptone agar, a

east (Smith, 1978). The organisms are found in feces of normal farm animals of the normal flora (Easton and resented in carrion and thus are used as feed, bedding, and sources of outbreaks in cattle (Egyed,

especially since lipase-negative organisms such as *C. botulinum* type G, *C. baratii*, and *C. butyricum* can produce neurotoxin. Isolated organisms can be fully characterized using methods described by Dowell and Hawkins (1974), Holdeman *et al.* (1977), and Dezfulian and Dowell (1980).

## Identification

Identifying botulinum toxin in cultures using mouse toxicity and neutralization tests on culture supernatants or filtrates, injecting into mice (approximately 0.1 ml) and identifying botulinum antitoxins. Mice die in 6–48 hr, depending on dose, before death. The toxin type is identified by neutralizing the toxicity. *In vitro* neutralizing numerous samples of toxin and identifying fractions in a chromatographic assay of the toxin and merits of this method are fully in Chapter 14.

## Organisms

Organisms from animal and food and soil samples are identified by neutralizing much of the competing flora. A differential characteristic lipase test (Dezfulian *et al.*, 1981; Mills and Dowell, 1977) covering heat-resistant spores. To identify Clostridium at lower temperature for heat treatment is sometimes necessary. Occasionally organisms because nontoxigenic, Clostridia are also present in high numbers. A method has been proposed to distinguish nontoxigenic organisms on agar by inoculating into chopped meat or serum (Hill *et al.*, 1977a); organisms are identified by extensions of the original specimen by Hatheway, 1988). Single colonies are identified on chopped meat–glucose–starch medium. Toxicity and neutralization tests should also be tested for toxin,

## IV. Organisms That Produce Tetanus Neurotoxin

### A. Toxins Produced by *Clostridium tetani*

The neurotoxin produced by *C. tetani* is referred to as tetanospasmin. Only one serologic type of tetanospasmin is known, and the organisms that produce it are phenotypically similar. Based on cellular antigens, nine bacterial agglutination types have been recognized (Coleman and Gunnison, 1928; Smith and Williams, 1984).

Physiologic characteristics of *C. tetani* strains are fairly uniform. Some strains of *C. tetani* that are toxigenic when initially isolated subsequently lose their toxigenicity (Fildes, 1925). A second toxin, tetanolysin, causes hemolysis on blood agar plates. It is doubtful that this toxin plays any role in the pathology of tetanus (Adams *et al.*, 1969).

### B. Cellular Morphology and Spores

*C. tetani* is usually a gram-negative rod that may retain some gram-positive staining in portions near the spore. The entire cell may be gram-positive in young cultures (less than 18 hr old). Unsporulated cells in cultures older than 24 hr usually appear gram-negative. Cell dimensions are  $0.5\text{--}1.7 \times 2.1\text{--}18.1 \mu\text{m}$ . Most strains are motile by means of peritrichous flagella (Cato *et al.*, 1986). Spores are usually in a terminal position and are more common in older cultures. Photomicrographs of representative strains are shown in Fig. 1 and 2. Spores generally survive moderate heating (75–80°C for 10 min) but usually are destroyed after heating at 100°C for 1 hr. Spores of some strains are more resistant, so that heating at 100°C for 4 hr or autoclaving for at least 10 min is required to ensure sterility (Adams *et al.*, 1969). Spores can survive exposure to alcohol, phenol, and even formaldehyde solutions. Aqueous and alcoholic iodine, glutaraldehyde, and hydrogen peroxide are some of the more effective sporicidal agents.

### C. Cultural and Physiologic Characteristics

*C. tetani* is a strict anaerobe. It will grow on the surface of solid media such as blood agar, peptone agar, and egg yolk agar only in an anaerobic environment



such as a Gas-Pak jar (BBL Laboratories, Cockeysville, Maryland)\* or in an anaerobe chamber with an atmosphere of 10% H<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub>. Optimal growth occurs at 37°C, while little or no growth takes place at 25°C or 42°C. Growth may not be visible until after 48 hr of incubation. Growth on agar is often apparent as a film rather than as discrete colonies because of swarming due to vigorous motility. Media containing 3–4% agar are more conducive to formation of discrete colonies. Colonies on blood agar are 4–6 mm in diameter, flat, translucent, gray with a matte surface, and show a narrow zone of clear (beta-type) hemolysis. Colonies have irregular and rhizoid margins (Cato *et al.*, 1986).

Growth in liquid medium does not require incubation in an anaerobic atmosphere. Dissolved oxygen can be driven out by heating tubed media in a boiling waterbath for 10 min. The broth is cooled before being inoculated. Metallic iron (filings or strips) or sodium thioglycollate help produce reducing conditions. *C. tetani* spores can germinate in media with an oxidation–reduction potential  $E_h$  of +580 mv, although vegetative growth does not occur unless the  $E_h$  is diminished. It has been shown that the  $E_h$  of liver broth diminishes after inoculation of spores sufficient for vegetative growth if the initial  $E_h$  is +300 mv or less, or even at +500 mv if the medium has a cover of liquid paraffin (Hachisuka *et al.*, 1982).

Most common biochemical tests are negative. No sugars are fermented; milk and other complex proteins are not digested. Neither lecithinase nor lipase is produced, and nitrate is not reduced. Gelatin is liquefied slowly, requiring perhaps 7 days for complete liquefaction. Hydrogen sulfide, DNase, and indole are produced. Gas-liquid chromatography of cultures in peptone-yeast extract with or without glucose (Lombard and Dowell, 1982) show butanol and acetic, propionic, and butyric acids as metabolic end-products (Holdeman *et al.*, 1977).

#### D. Genetic Studies

Nakamura *et al.* (1979) compared 10 strains of *C. tetani* and found they were all related to each other by 89% DNA sequence similarity. Three nontoxicogenic strains (*C. tetani*-like strains that resembled *C. tetani* in all respects except toxicogenicity) were closely related (85–93% similarity). *C. tetani* could be clearly distinguished from *C. cochlearium* and *C. tetanomorphum*, which are nontoxicogenic, by DNA sequence differences. Oddly, one *C. tetani*-like strain that was toxicogenic when isolated but lost toxicogenicity after treatment at 100°C for 30 min was more closely related to *C. cochlearium* than to *C. tetani*. Cato *et al.* (1986) distinguished between *C. cochlearium* and *C. tetani* on the basis that the former is nontoxicogenic and is weaker or negative in the liquefaction of gelatin. Thus it

#### I. Bacterial Sources of Clostridi

seems very difficult to distinguish *Cochlearium* without DNA analysis. These species may be distinct (Cato *et al.*, 1986). Comparing a sufficient number of strains, *C. tetanomorphum* has been found to be a distinct species. Available reference strains do not include *C. tetanomorphum* species (Cato *et al.*, 1986). *C. tetanomorphum* while *C. cochlearium* has 27

#### E. Bacteriophages

Based on the findings of the *Corynebacterium diphtheriae* toxin production by *C. tetani*, mitomycin C induced lysis of presumptive evidence of lysogenicity, the possibly induced phages in two of the strains was verified (1967b). Hara *et al.* (1977) cultivated it with acridine orange, N-nitrophenol, and ultraviolet light; they found that the strains possess the same bacteriophages. Studies on toxicogenic strains of *C. tetani* revealed that single large plasmid (Lai *et al.*, 1984) oligonucleotides coding for a toxin reaction showed that the toxin gene sequence and structure (Cato *et al.*, 1986).

#### F. H

*C. tetani* is commonly found in soil. Surveys have been made. In the United States have yielded 30–42% of *C. tetani* has also been commonly found. Meyer (1926), Ten Broeck (1926) found the organism in about 25–30% of soil in China, and England, respectively. Naturally acquired immunity in humans and animals (Veronesi *et al.*, 1977). Serum of experimental animals (Wells and Balish, 1977).

\*Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

keys-ville, Maryland)\* or in an atmosphere of 5% H<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub>. No growth takes place at 25°C or at 4°C of incubation. Growth on agar slants and colonies because of swarming on 4% agar are more conducive to spreading. Colonies on agar are 4–6 mm in diameter, and show a narrow zone of clear liquid and rhizoid margins (Cato *et al.*,

1986). For anaerobic incubation in an anaerobic jar, tubes of media in a water bath at 37°C before being inoculated. Glycollate help produce reducing media with an oxidation–reduction potential. Growth does not occur unless the E<sub>h</sub> of liver broth diminishes after incubation if the initial E<sub>h</sub> is +300 mv. Media has a cover of liquid paraffin

is not fermentative. No sugars are fermented; gelatin is not. Neither lecithinase nor lipase is produced. Media is liquefied slowly, requiring reduction of oxygen sulfide, DNase, and indole production. Cultures in peptone-yeast extract (1982) show butanol and acetic acid products (Holdeman *et al.*, 1977).

## Diagnosis

*C. tetani* and found they were all very similar. Three nontoxicogenic *C. tetani* in all respects except motility. *C. tetani* could be clearly distinguished from *C. tetanomorphum*, which are nontoxicogenic. One *C. tetani*-like strain that was killed by treatment at 100°C for 30 min was not *C. tetani*. Cato *et al.* (1986) distinguished *C. tetani* on the basis that the former does not liquefy gelatin. Thus it

is not an official endorsement by the Public Health Service.

seems very difficult to distinguish between nontoxicogenic *C. tetani* and *C. cochlearium* without DNA comparison. Soluble cellular proteins of the two species may be distinct (Cato *et al.*, 1982), but this has not been verified by comparing a sufficient number of strains identified by DNA studies. The species *C. tetanomorphum* has been omitted from Bergey's Manual because the available reference strains do not have the characteristics originally described for the species (Cato *et al.*, 1986). *C. tetani* has 25–26 mol% G+C content in its DNA, while *C. cochlearium* has 27–28% (Cato *et al.*, 1986).

## E. Bacteriophages, Plasmids, and Toxicogenicity

Based on the findings of the relation of a lysogenic phage to toxicogenicity of *Corynebacterium diphtheriae*, researchers sought evidence for a phage role in toxin production by *C. tetani*. Prescott and Altenbern (1967a) found that mitomycin C induced lysis of all seven strains of *C. tetani* tested. This provided presumptive evidence of lysogenic phages, but there was no indication that any of the possibly induced phages were related to toxicogenicity. The presence of phages in two of the strains was verified by electron microscopy (Prescott and Altenbern, 1967b). Hara *et al.* (1977) cured a strain of *C. tetani* of its toxicogenicity by treating it with acridine orange, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, rifampicin, and ultraviolet light; they found that the nontoxicogenic derivative strains appeared to possess the same bacteriophages as the toxicogenic parent strain. Subsequently, studies on toxicogenic strains of *C. tetani* and nontoxicogenic derivatives of three toxicogenic strains revealed that loss of toxicogenicity was correlated with loss of a single large plasmid (Laird *et al.*, 1980). Further investigation using oligonucleotides coding for an amino-terminal amino acid sequence in hybridization reactions showed that the gene for the toxin is on the plasmid (Finn *et al.*, 1984). The entire nucleotide sequence of the plasmid toxin gene and the amino acid sequence and structure of the toxin molecule have since been reported (Eisel *et al.*, 1986).

## F. Habitat and Distribution

*C. tetani* is commonly found in soil samples in all parts of the world where surveys have been made. Surveys in Japan, Canada, Brazil, and the United States have yielded 30–42% positive samples (Smith and Williams, 1984). *C. tetani* has also been commonly found in human and animal feces. Bauer and Meyer (1926), Ten Broeck and Bauer (1922), and Tulloch (1919–1920) found the organism in about 25–35% of human fecal samples in the United States, China, and England, respectively. Intestinal carriage may be responsible for naturally acquired immunity to tetanus toxin in some nonimmunized humans and animals (Veronesi *et al.*, 1983). Antitoxin, but no toxin, has been found in the serum of experimental animals that have been intestinally colonized with *C. tetani* (Wells and Balish, 1983).

### G. Toxin Detection and Identification

Tetanus toxin can be demonstrated by intramuscular (im) injection of the test sample into a test animal. For example, 0.1 ml of a culture supernatant is injected into the rear leg muscle of a mouse; the mouse is observed for several days for the development of rigid paralysis in the injected muscle and the progression of the paralysis into the rest of the musculature of the lower body. A second mouse injected with a mixture of 0.1 ml of culture supernatant and 0.1 ml of tetanus antitoxin should show no paralytic signs. Intraperitoneal (ip) injection of moderate to large amounts of toxin may kill the mice before signs of tetanus can be recognized; smaller amounts cause a distended, rigid abdominal area with gradual development of a generalized tetanus. Injection of samples mixed with antitoxin should have no effect, unless the amount of toxin overwhelms the neutralizing capacity of the antitoxin. Beland and Rossier (1973) recommend testing cultures for toxigenicity by mixing equal volumes of broth culture and 2% calcium chloride and injecting 0.5 ml of this mixture at the base of the tail of each mouse. Mice pretreated by ip injection of 1500 IU of tetanus antitoxin 1 hr before challenge serve as controls. Test animals should be properly disposed of as soon as a conclusive judgment can be made on the basis of paralytic signs.

### H. Isolation of Organisms

Tetanus is not usually confirmed bacteriologically because the signs of this condition provide unmistakable evidence for the diagnosis. Often the infected wound is not obvious, and when cultured, the causative organism is not recovered. Most of the strains of *C. tetani* have been isolated from soil. *C. tetani* can often be isolated by culture from material that does not show typical "drumstick" organisms upon microscopic examination (Adams *et al.*, 1969). Pieces of dead tissue taken from deep in wounds are ideal specimens for culture. Swabs of the wound offer less chance for success. Soil samples can be cultured using the same methods used for wound samples.

The medium of choice for culturing *C. tetani* is chopped meat medium (Dowell *et al.*, 1977a). Selective advantage is taken of the spores' resistance to heat, although heat treatment may result in decreased toxicity of the subsequent cultures and increased numbers of nontoxigenic isolates. Adams *et al.* (1969) recommend inoculation of three broth cultures, heating one at 80°C for 15 min, heating the second for 5 min, and not heating the third. Beland and Rossier (1973) recommend inoculating soil samples after suspending them in sterile saline: one portion of the suspended sample is inoculated after a 10 min treatment at 60°C; another portion is inoculated without treatment. Both cultures after 48 hr incubation at 37°C are streaked onto a half (or less) of a blood agar plate and incubated anaerobically at 37°C and observed at 24 and 48 hr for swarming growth. The bacterial growth at the leading edge of the swarming growth is picked and inoculated into chopped meat medium. Distinct colonies of the

### I. Bacterial Sources of Clostridia

swarming organism may be one of some of the swarming growth (Adams *et al.*, 1977a; Smith and W. microscopically, tested for toxigenicity characteristics (Dowell and Hawk).

### V. Fi

In view of the unusual features caused by botulism, organisms are isolated when possible. Reports of tetanus antitoxin (Berger *et al.*, 1973) possible existence of strains neutralized by the antibodies.

The observation of *proteus* *paratyphi* and *C. butyricum* raises the possibility of organisms that produce the toxin, which may have been called *C. botulinum*. Thus, the study of toxigenicity seems to deserve

- Adams, E.B., Laurence, D.R., and Hatheway, C.L. (1977a). Oxford.
- Aureli, P., Fenicia, L., Pasoli, L., and Hatheway, C.L. (1986). Two strains of *Clostridium butyricum* in Italy. *California J. Infect. Dis.* **38**.
- Bauer, J.H., and Meyer, K.F. (1973). *California J. Infect. Dis.* **38**.
- Beland, S., and Rossier, E. (1973). *le sol des Cantons de l'Est de la Suisse* **1518**.
- Bengtson, I.A. (1922). Preliminary report on the larvae of *Lucilia caesar*. *Pulmonary Hygiene* **136**, U.S. P. D.C.
- Berger, S.A., Cherubin, C.E., and Burke, G.S. (1919). Notes on the isolation of *Clostridium botulinum* from the soil. *Microbiol. Rev.* **28**.
- Bytchenko, B. (1981). *Microbiology* (R. Veronesi, ed.), pp. 28-30.
- Cato, E.P., Hash, D.E., Holder, and Hatheway, C.L. (1977b). A study of *Clostridium* species

## Identification

cular (im) injection of the test a culture supernatant is injected is observed for several days for muscle and the progression of the lower body. A second mouse pernatant and 0.1 ml of tetanus ritoneal (ip) injection of moderate before signs of tetanus can be rigid abdominal area with gradual of samples mixed with antitoxin overwhelms the neutralizing (73) recommend testing cultures broth culture and 2% calcium the base of the tail of each mouse. of tetanus antitoxin 1 hr before be properly disposed of as soon basis of paralytic signs.

## Organisms

cally because the signs of this the diagnosis. Often the infected a causative organism is not been isolated from soil. *C. tetani* at does not show typical "drum- (Adams *et al.*, 1969). Pieces of specimens for culture. Swabs of amplex can be cultured using the

*tetani* is chopped meat medium taken of the spores' resistance to reased toxicity of the subsequent ic isolates. Adams *et al.* (1969) heating one at 80°C for 15 min, g the third. Beland and Rossier after suspending them in sterile inoculated after a 10 min treatment eatment. Both cultures after 48 hr r less) of a blood agar plate and l at 24 and 48 hr for swarming edge of the swarming growth is edium. Distinct colonies of the

swarming organism may be obtained by streaking the original broth culture or some of the swarming growth on "stiff" blood agar containing 4% agar (Dowell *et al.*, 1977a; Smith and Williams, 1984). Isolated organisms are observed microscopically, tested for toxigenicity, and examined for their physiologic characteristics (Dowell and Hawkins, 1974; Holdeman *et al.*, 1977).

## V. Final Considerations

In view of the unusual features of some of the toxigenic organisms that have caused botulism, organisms responsible for tetanus cases should be isolated when possible. Reports of tetanus in patients with demonstrable circulating tetanus antitoxin (Berger *et al.*, 1978; Passen and Andersen, 1986) suggest the possible existence of strains that produce a type of tetanospasmin that is not neutralized by the antibodies engendered by the immunizing toxoid.

The observation of production of botulinum neurotoxin in strains of *C. baratii* and *C. butyricum* raises the question of whether it is practical to call all organisms that produce the toxin *C. botulinum*. Additionally, nontoxigenic organisms, which may have been derived from toxigenic strains, cannot be properly called *C. botulinum*. Thus, strict classification of *Clostridium* species on the basis of toxigenicity seems to deserve reconsideration.

## References

- Adams, E.B., Laurence, D.R., and Smith, J.W.G. (1969). "Tetanus." Blackwell, Oxford.
- Aureli, P., Fenicia, L., Pasolini, B., Gianfranceschi, M., McCroskey, L.M., and Hatheway, C.L. (1986). Two cases of type E infant botulism caused by neurotoxicogenic *Clostridium butyricum* in Italy. *J. Infect. Dis.* **154**, 207-211.
- Bauer, J.H., and Meyer, K.F. (1926). Human intestinal carriers of tetanus spores in California. *J. Infect. Dis.* **38**, 295-305.
- Beland, S., and Rossier, E. (1973). Isolement et identification de *Clostridium tetani* dans le sol des Cantons de l'Est de la province de Quebec. *Can. J. Microbiol.* **19**, 1513-1518.
- Bengtson, I.A. (1922). Preliminary note on a toxin producing anaerobe isolated from the larvae of *Lucilia caesar*. *Public Health Rep.* **37**, 164-170.
- Bengtson, I.A. (1924). Studies on organisms concerned as causative factors in botulism. *Hyg. Lab. Bull.* **136**, U.S. Public Health Service, Govt Printing Office, Washington, D.C.
- Berger, S.A., Cherubin, C.E., Nelson, S., and Levine, L. (1978). Tetanus despite preexisting antitetanus antibody. *J. Am. Med. Assoc.* **240**, 769-770.
- Burke, G.S. (1919). Notes on *Bacillus botulinus*. *J. Bacteriol.* **4**, 555-565.
- Bytchenko, B. (1981). Microbiology of tetanus: In, "Tetanus: Important New Concepts" (R. Veronesi, ed.), pp. 28-39. Excerpta Medica, Amsterdam.
- Cato, E.P., Hash, D.E., Holdeman, L.V., and Moore, W.E.C. (1982). Electrophoretic study of *Clostridium* species. *J. Clin. Microbiol.* **15**, 688-702.

- Cato, E.P., George, W.L., and Finegold, S.M. (1986). Genus *Clostridium*. In "Bergey's Manual of Systematic Bacteriology, Vol. 2 (Sneath, P.H.A., Mair, N.S., Sharpe, M.E., and Holt, J.G., eds.), pp. 1141-1200. Williams and Wilkins, Baltimore, Maryland.
- Ciccarelli, A.S., and Gimenez, D.F. (1971). Estudio serologico de toxinas de *Clostridium botulinum* tipo A y cepa 84. *Rev. Latino am. Microbiol.* **13**, 64-67.
- Coleman, G.E., and Gunnison, J.B. (1928). Serologic types of *Clostridium tetani*. *J. Infect. Dis.* **43**, 184-188.
- Decker, K., Jungermann, K., and Thauer, R.K. (1970). Energy production in anaerobic organisms. *Angew. Chemie (Internat. Ed.)* **9**, 138-158.
- Dezfulian, M., and Dowell, V.R., Jr. (1980). Cultural and physiological characteristics and antimicrobial susceptibility of *Clostridium botulinum* isolates from foodborne and infant botulism cases. *J. Clin. Microbiol.* **11**, 604-609.
- Dezfulian, M., McCroskey, L.M., Hatheway, C.L., and Dowell, V.R., Jr. (1981). Selective medium for isolation of *Clostridium botulinum* from human feces. *J. Clin. Microbiol.* **13**, 526-531.
- Dolman, C.E., and Chang, E. (1972). Bacteriophages of *Clostridium botulinum*. *Can. J. Microbiol.* **18**, 67-76.
- Dowell, V.R., Jr., and Hawkins, T.M. (1974). Laboratory methods in anaerobic bacteriology. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Georgia.
- Dowell, V.R., Jr., Lombard, G.L., Thompson, F.S., and Armfield, A.Y. (1977a). Media for isolation, characterization, and identification of obligately anaerobic bacteria. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, GA. 46pp.
- Dowell, V.R., Jr., McCroskey, L.M., Hatheway, C.L., Lombard, G.L., Hughes, J.M., and Merson, M.H. (1977b). Coproexamination for botulinum toxin and *Clostridium botulinum*. *J. Am. Med. Assoc.* **238**, 1829-1839.
- Easton, E.J., and Meyer, K.F. (1924). Occurrence of *Bacillus botulinus* in human and animal excreta. *J. Infect. Dis.* **35**, 207-212.
- Egyed, M.N. (1987). Outbreaks of botulism in ruminants associated with ingestion of feed containing poultry waste. In "Avian Botulism" (M.W. Eklund and V.R. Dowell, Jr., eds.), pp. 371-380. Thomas, Springfield, Illinois.
- Eisel, V., Jarausch, W., Goretski, K., Henschen, A., Engels, J., Weller, U., Hudel, M., Habermann, E., and Neimann, H. (1986). Tetanus toxin: primary structure, expression in *E. coli* and homology with botulinum toxins. *EMBO J.* **5**, 2495-2502.
- Eklund, M.W., and Poysky, F.T. (1974). Interconversion of type C and D strains of *Clostridium botulinum* by specific bacteriophages. *Appl. Microbiol.* **27**, 251-258.
- Eklund, M.W., Wieler, D.I., and Poysky, F.T. (1967). Outgrowth and toxin production of non-proteolytic type B *Clostridium botulinum* at 3.3-5.6°C. *J. Bacteriol.* **93**, 1461-1462.
- Eklund, M.W., Poysky, F.T., and Boatman, E.S. (1969). Bacteriophages of *Clostridium botulinum* types A, B, E, and F non-toxigenic strains resembling type E. *J. Virol.* **3**, 270-274.
- Eklund, M.W., Poysky, F.T., Meyers, J.A., and Pelroy, G.A. (1974). Interspecies conversion of *Clostridium botulinum* type C to *Clostridium novyi* type A by bacteriophage. *Science* **186**, 456-458.
- Ferreira, J.L., Hamdy, M.K., Zapatka, F.A., and Hebert, W.O. (1983). Immunodiffusion method for detection of C
- 5, 87-94.
- Fildes, P. (1925). Tetanus: Aggl
- 6, 91-98.
- Finn, C.W., Silver, R.P., Habig
1984. The structural gene for
- 884.
- Gimenez, D.F., and Ciccarelli,
- ulinum. *Zentralbl. Bacteriol.*,
- Gimenez, D.F., and Ciccarelli,
- Zentralbl. Bacteriol.*, *I. Abt.*
- Gunnison, J.B., and Meyer, K.F
- Clostridium botulinum* and *pa*
- Gunnison, J.B., Cummings, J.R.
- type E. *Proc. Soc. Exp. Biol.*
- Hachisuka, Y., Suzuki, I., Morik
- reduction potential on spore
- Clostridium tetani*, *Clostridium*
- 26**, 803-811.
- Hall, J.D., McCroskey, L.M., P
- an organism resembling *Clo*
- from an infant with botulism
- Hara, T., Matsuda, M., and Yo
- toxigenic derivatives of a str
- Hatheway, C.L. (1988). Botuli
- Principles and Practice." Vo
- Balows, W.J. Hausler, Jr., M
- Verlag, New York.
- Hatheway, C.L., and McCrosk
- diagnosis of infant botulism: I
- 2338.
- Hatheway, C.L., McCroskey, I
- Atypical toxin variant of *Cl*
- ulism. *J. Clin. Microbiol.* **14**
- Hauschild, A.H.W. (1989). *Clos*
- (M.P. Doyle, ed.), pp. 112-
- Hazen, E.L. (1937). A strain of
- Dis.* **60**, 260-264.
- Hewitt, J., and Morris, J.G. (19
- bacteria. *FEBS Lett.* **50**, 315
- Holdeman, L.V., Cato, E.P., a
- manual," 4th ed. Virginia I
- Virginia.
- Inoue, K., and Iida, H. (1968).
- 537-540.
- Jansen, B.C. (1987). *Clostridi*
- taxonomic position. In: "A
- eds.). pp. 123-132. Thomas

- 5). Genus *Clostridium*. In "Bergey's Manual of Systematic Bacteriology," 8th ed., P.H.A. Mair, N.S. Sharpe, Williams and Wilkins, Baltimore, 1974, pp. 111-112.
- 6). Serological and epidemiological studies of *Clostridium botulinum* type E. *J. Clin. Microbiol.* **13**, 64-67.
- 7). Serological types of *Clostridium tetani*. *J. Clin. Microbiol.* **13**, 64-67.
- 8). Energy production in anaerobic bacteria. *J. Biol. Chem.* **215**, 8-158.
- 9). Cultural and physiological characteristics of *Clostridium botulinum* isolates from foodborne and environmental sources. *J. Clin. Microbiol.* **14**, 604-609.
- 10). L., and Dowell, V.R., Jr. (1981). Isolation of *Clostridium botulinum* from human feces. *J. Clin. Microbiol.* **23**, 111-113.
- 11). Serological types of *Clostridium botulinum*. *Can. J. Microbiol.* **27**, 111-113.
- 12). Laboratory methods in anaerobic bacteriology. *Public Health Service, Centers for Disease Control, Atlanta, Georgia*, 1974.
- 13). F.S., and Armfield, A.Y. (1977a). Isolation of obligately anaerobic bacteria. *Public Health Service, Centers for Disease Control, Atlanta, Georgia*, 1977.
- 14). C.L., Lombard, G.L., Hughes, J.M., and Morris, J.G. (1981). Isolation of *Bacillus botulinus* in human and animal feces. *J. Clin. Microbiol.* **23**, 111-113.
- 15). Factors associated with ingestion of botulinum toxin (M.W. Eklund and V.R. Dowell, Jr., eds.). *Public Health Service, Centers for Disease Control, Atlanta, Georgia*, 1977.
- 16). J., Engels, J., Weller, U., Hudel, M., and Morris, J.G. (1981). Botulinum toxin: primary structure, exotoxins. *EMBO J.* **5**, 2495-2502.
- 17). Conversion of type C and D strains of *Clostridium botulinum* to type E. *Appl. Microbiol.* **27**, 251-258.
- 18). (1967). Outgrowth and toxin production of *Clostridium botulinum* at 3.3-5.6°C. *J. Bacteriol.* **93**, 111-113.
- 19). (1969). Bacteriophages of *Clostridium botulinum* strains resembling type E. *J. Virol.* **3**, 111-113.
- 20). J. Pelroy, G.A. (1974). Interspecies transfer of *Clostridium novyi* type A by bacteriophage. *J. Virol.* **45**, 111-113.
- 21). Hebert, W.O. (1983). Immunodiffusion method for detection of *Clostridium botulinum* types A, B, E, and F. *J. Food Saf.* **5**, 87-94.
- 22). Fildes, P. (1925). Tetanus: Agglutination and toxicity of *B. tetani*. *Brit. J. Exp. Pathol.* **6**, 91-98.
- 23). Finn, C.W., Silver, R.P., Habig, W.H., Hardegree, M.C., Zon, G., and Garon, C.F. (1984). The structural gene for tetanus neurotoxin is on a plasmid. *Science* **224**, 881-884.
- 24). Gimenez, D.F., and Ciccarelli, A.S. (1970a). Studies on strain 84 of *Clostridium botulinum*. *Zentralbl. Bakteriol., I. Abt. Orig.* **215**, 212-220.
- 25). Gimenez, D.F., and Ciccarelli, A.S. (1970b). Another type of *Clostridium botulinum*. *Zentralbl. Bakteriol., I. Abt. Orig.* **215**, 221-224.
- 26). Gunnison, J.B., and Meyer, K.F. (1929). Cultural study of an international collection of *Clostridium botulinum* and *parabotulinum*. *J. Infect. Dis.* **45**, 119-134.
- 27). Gunnison, J.B., Cummings, J.R., and Meyer, K.F. (1936-1937). *Clostridium botulinum* type E. *Proc. Soc. Exp. Biol. Med.* **35**, 278-280.
- 28). Hachisuka, Y., Suzuki, I., Morikawa, K., and Maeda, S. (1982). The effect of oxidation-reduction potential on spore germination, outgrowth, and vegetative growth of *Clostridium tetani*, *Clostridium butyricum*, and *Bacillus subtilis*. *Microbiol. Immunol.* **26**, 803-811.
- 29). Hall, J.D., McCroskey, L.M., Pincomb, B.J., and Hatheway, C.L. (1985). Isolation of an organism resembling *Clostridium baratii* which produces type F botulinum toxin from an infant with botulism. *J. Clin. Microbiol.* **21**, 654-655.
- 30). Hara, T., Matsuda, M., and Yoneda, M. (1977). Isolation and some properties of non-toxic derivatives of a strain of *Clostridium tetani*. *Biken J.* **20**, 105-115.
- 31). Hatheway, C.L. (1988). Botulism. In "Laboratory Diagnosis of Infectious Diseases: Principles and Practice." Vol. I, "Bacterial, Mycotic, and Parasitic Diseases" (A. Balows, W.J. Hausler, Jr., M. Ohashi, and A. Tenover, eds.), pp. 111-133. Springer-Verlag, New York.
- 32). Hatheway, C.L., and McCroskey, L.M. (1987). Examination of feces and serum for diagnosis of infant botulism: Experience in 336 patients. *J. Clin. Microbiol.* **25**, 2334-2338.
- 33). Hatheway, C.L., McCroskey, L.M., Lombard, G.L., and Dowell, V.R., Jr. (1981). Atypical toxin variant of *Clostridium botulinum* type B associated with infant botulism. *J. Clin. Microbiol.* **14**, 607-611.
- 34). Hauschild, A.H.W. (1989). *Clostridium botulinum*. In "Foodborne Bacterial Pathogens" (M.P. Doyle, ed.), pp. 112-189. Dekker, New York.
- 35). Hazen, E.L. (1937). A strain of *B. botulinus* not classified as type A, B, or C. *J. Infect. Dis.* **60**, 260-264.
- 36). Hewitt, J., and Morris, J.G. (1975). Superoxide dimutase in some obligately anaerobic bacteria. *FEBS Lett.* **50**, 315-318.
- 37). Holdeman, L.V., Cato, E.P., and Moore, W.E.C., eds. (1977). "Anaerobe laboratory manual," 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
- 38). Inoue, K., and Iida, H. (1968). Bacteriophages of *Clostridium botulinum*. *J. Virol.* **2**, 537-540.
- 39). Jansen, B.C. (1987). *Clostridium botulinum* type C, its isolation, identification and taxonomic position. In "Avian Botulism" (M.W. Eklund and V.R. Dowell, Jr., eds.), pp. 123-132. Thomas, Springfield, Illinois.

- Johnston, R., Harmon, S., and Kautter, D. (1962). Method to facilitate the isolation of *Clostridium botulinum* type E. *J. Bacteriol.* **88**, 1521-1522.
- Kobel, T., and Marti, M.C. (1985). Decouverte du bacille de tetanos (1884). *Rev. Med. Suisse Romande* **105**, 547-556.
- Kushnir, E.D., Burn, T.M., and Paykina, S. Sh. (1937). Istochniki infitsirovaniya krasnoy ribi mikrobom botulizma (Sources of *Bacillus botulinus* infection of sturgeons). *Zh. Mikrobiol. Epidemiol. Immunobiol.* **19**, 80-85.
- Laird, W.J., Aaronson, W., Silver, R.P., Habig, W.H., and Hardegree, M.C. (1980). Plasmid-associated toxigenicity in *Clostridium tetani*. *J. Infect. Dis.* **142**, 623.
- Landman, G. (1904). Ueber die Ursache der Darmstadter Bohnen Vergiftung. *Hyg. Rundsch.* **14**, 449-452.
- Lee, W.H., and Riemann, H. (1970a). The genetic relatedness of proteolytic *Clostridium botulinum* strains. *J. Gen. Microbiol.* **64**, 85-90.
- Lee, W.H., and Riemann, H. (1970b). Correlation of toxic and non-toxic strains of *Clostridium botulinum* by DNA composition and homology. *J. Gen. Microbiol.* **60**, 117-123.
- Leuchs, J. (1910). Beitrage zur kenntnis des toxins und antitoxins des *Bacillus botulinus*. *Z. Hyg. Infektionskrankh.* **65**, 55-84.
- Lombard, G.L., and Dowell, V.R., Jr. (1982). Gas-liquid chromatography analysis of the acid products of bacteria. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Georgia.
- Lynt, R.K., Solomon, H.M., and Kautter, D.A. (1984). Heat resistance of *Clostridium botulinum* type G in phosphate buffer. *J. Food. Prot.* **47**, 463-466.
- McCroskey, L.M., and Hatheway, C.L. (1988). Laboratory findings in four cases of adult botulism that suggest colonization of the intestinal tract. *J. Clin. Microbiol.* **26**, 1052-1054.
- McCroskey, L.M., Hatheway, C.L., Fenicia, L., Pasolini, B., and Aureli, P. (1986). Characterization of an organism that produces Type E botulin toxin but which resembles *Clostridium butyricum* from the feces of an infant with type E botulism. *J. Clin. Microbiol.* **23**, 201-202.
- Merson, M.H., and Dowell, V.R., Jr. (1973). Epidemiologic, clinical and laboratory aspects of wound botulism. *N. Engl. J. Med.* **289**, 1005-1010.
- Meyer, K.F., and Gunnison, J.B. (1928). *C. botulinum* type D sp. Nov. *Proc. Soc. Exp. Biol. Med.* **26**, 88-89.
- Midura, T.F., and Arnon, S.S. (1976). Infant botulism: identification of *Clostridium botulinum* and its toxins in faeces. *Lancet* **2**, 934-936.
- Mills, D.C., Midura, T.F., and Arnon, S.S. (1985). Improved selective medium for the isolation of lipase-positive *Clostridium botulinum* from feces of human infants. *J. Clin. Microbiol.* **21**, 947-950.
- Moller, V., and Scheibel, I. (1960). Preliminary report on the isolation of an apparently new type of *Cl. botulinum*. *Acta Pathol. Microbiol. Scand.* **48**, 80.
- Morris, J.G. (1975). The physiology of obligate anaerobiosis. *Adv. Microb. Physiol.* **12**, 169-245.
- Nakamura, S., Okado, I., Nakashio, S., and Nishida, S. (1977). *Clostridium sporogenes* isolates and their relationship to *C. botulinum* based on deoxyribonucleic acid reassociation. *J. Gen. Microbiol.* **100**, 395-401.
- Nakamura, S., Okado, I., Abe, T., and Nishida, S. (1979). Taxonomy of *Clostridium tetani* and related species. *J. Gen. Microbiol.* **113**, 29-35.

## 1. Bacterial Sources of Clostrid

- Nakamura, S., Kimura, I., Yano, T. (1977). Relationships among *Clostridium botulinum* type E and *Clostridium botulinum* type C. *J. Infect. Dis.* **137**, 100-104.
- Passen, E.L., and Andersen, B. (1985). Botulin toxin-neutralizing antibody. *J. Infect. Dis.* **151**, 100-104.
- Pickett, J., Berg, B., Chaplin, R. (1985). Botulism in infancy: clinical features. *J. Infect. Dis.* **151**, 772.
- Prescott, L.M., and Altenbern, L.M. (1978). *Bacteriol.* **93**, 1220-1226.
- Prescott, L.M., and Altenbern, L.M. (1978). Strains of *Clostridium tetani*. *J. Infect. Dis.* **137**, 100-104.
- Seddon, H.R. (1922). Bulbar paralysis. *Comp. Pathol. Ther.* **35**, 14.
- Simpson, L. L. (1982). A comparison of *Clostridium botulinum* type C<sub>1</sub> and type C<sub>2</sub>. *J. Infect. Dis.* **145**, 100-104.
- Smart, J.L., Jones, T.O., Cleaveland, J.E. (1977). Associated type C botulism. *J. Infect. Dis.* **137**, 100-104.
- Smith, L.D.S. (1977). "Botulism." Springfield, Illinois.
- Smith, L.D.S. (1978). The occurrence of botulism in the United States. *Health L.* **10**, 100-104.
- Smith, L.D.S., and Williams, J. (1978). Thomas, Springfield, Illinois.
- Sonnabend, O., Sonnabend, W. (1981). Isolation of *Clostridium botulinum* toxin in humans: report. *J. Infect. Dis.* **143**, 22-27.
- Strom, M.S., Eklund, M.W., and Suen, J.C. (1986). *ulium* and related *Clostridium* species. Ph.D. dissertation.
- Suen, J.C. (1986). DNA homology of *Clostridium* clostridial species. Ph.D. dissertation.
- Suen, J.C., Hatheway, C.L., and Suen, J.C. (1986). Relatedness to study taxonomic relationships among similar clostridial species (Clostridium). *J. Infect. Dis.* **153**, 100-104.
- Suen, J.C., Hatheway, C.L., and Suen, J.C. (1986). Confirmation of identities of *Clostridium butyricum* implicated as agent of botulism. *J. Infect. Dis.* **153**, 2192.
- Ten Broeck, C., and Bauer, J. (1978). Botulism in man. *J. Exp. Med.* **36**, 100-104.
- Tulloch, W.J. (1919-1920). Food poisoning of the war office. *J. Hyg.* **19**, 100-104.
- van Ermengem, E. (1897). Ueber den Botulismus. *Z. Hyg. Rev. Infect. Dis.* **1**, 701-710.

- Method to facilitate the isolation of 1521-1522.
- bacille de tetanos (1884). *Rev. Med.*
- . (1937). Istochniki infitirovaniya *illus botulinus* infection of sturgeons). 35.
- V.H., and Hardegree, M.C. (1980). *tani*. *J. Infect. Dis.* **142**, 623.
- mstadter Bohnen Vergiftung. *Hyg.*
- relatedness of proteolytic *Clostridium* J.
- m of toxic and non-toxic strains of d homology. *J. Gen. Microbiol.* **60**,
- and antitoxins des *Bacillus botulinus*.
- as-liquid chromatography analysis of f Health and Human Services, Public lanta, Georgia.
- (1984). Heat resistance of *Clostridium* . *Prot.* **47**, 463-466.
- Laboratory findings in four cases of ntestinal tract. *J. Clin. Microbiol.* **26**,
- Pasolini, B., and Aureli, P. (1986). s Type E botulinal toxin but which of an infant with type E botulism. *J.*
- pidemiologic, clinical and laboratory 1989, 1005-1010.
- inum type D sp. Nov. *Proc. Soc. Exp.*
- otulism: identification of *Clostridium* 34-936.
- i). Improved selective medium for the um from feces of human infants. *J.*
- report on the isolation of an apparently biol. *Scand.* **48**, 80.
- aerobiosis. *Adv. Microb. Physiol.* **12**,
- da, S. (1977). *Clostridium sporogenes* based on deoxyribonucleic acid reas-
- S. (1979). Taxonomy of *Clostridium* **113**, 29-35.
- Nakamura, S., Kimura, I., Yamakawa, K., and Nichida, S. (1983). Taxonomic relationships among *Clostridium novyi* types A and B, *Clostridium haemolyticum* and *Clostridium botulinum* type C. *J. Gen. Microbiol.* **129**, 1473-1479.
- Passen, E.L., and Andersen, B.R. (1986). Clinical tetanus despite a protective level of toxin-neutralizing antibody. *J. Am. Med. Assoc.* **255**, 1171-1173.
- Pickett, J., Berg, B., Chaplin, E., and Brunstetter-Shafer, M.A. (1976). Syndrome of botulism in infancy: clinical and electrophysiologic study. *N. Engl. J. Med.* **295**, 770-772.
- Prescott, L.M., and Altenbern, R.A. (1967a). Inducible lysis in *Clostridium tetani*. *J. Bacteriol.* **93**, 1220-1226.
- Prescott, L.M., and Altenbern, R.A. (1967b). Detection of bacteriophages from two strains of *Clostridium tetani*. *J. Virol.* **1**, 1085-1086.
- Seddon, H.R. (1922). Bulbar paralysis in cattle due to the action of a toxicogenic bacillus with a discussion of the relationship of the condition to forage poisoning (botulism). *J. Comp. Pathol. Ther.* **35**, 147-190.
- Simpson, L. L. (1982). A comparison of the pharmacological properties of *Clostridium botulinum* type C<sub>1</sub> and type C<sub>2</sub> toxins. *J. Pharmacol. Exp. Ther.* **223**, 695-701.
- Smart, J.L., Jones, T.O., Clegg, F.G., and McMurtry, M.J. (1987). Poultry waste associated type C botulism in cattle. *Epidem. Inf.* **98**, 73-79.
- Smith, L.D.S. (1977). "Botulism: The Organism, Its Toxins, the Disease." Thomas, Springfield, Illinois.
- Smith, L.D.S. (1978). The occurrence of *Clostridium botulinum* and *C. tetani* in the soil of the United States. *Health Lab. Sci.* **15**, 74-80.
- Smith, L.D.S., and Williams, B.L. (1984). "The Pathogenic Anaerobic Bacteria." Thomas, Springfield, Illinois.
- Sonnabend, O., Sonnabend, W., Heinzle, R., Sigrist, T., Dirnhofer, R., and Krech, U. (1981). Isolation of *Clostridium botulinum* type G and identification of type G botulinal toxin in humans: report of five sudden unexpected deaths. *J. Infect. Dis.* **143**, 22-27.
- Strom, M.S., Eklund, M.W., and Poysky, F.T. (1984). Plasmids in *Clostridium botulinum* and related *Clostridium* species. *Appl. Environ. Microbiol.* **48**, 956-963.
- Suen, J.C. (1986). DNA homology studies on *Clostridium botulinum* and related clostridial species. Ph.D. diss., Univ. of North Carolina, Chapel Hill, North Carolina.
- Suen, J.C., Hatheway, C.L., Steigerwalt, A.G., and Brenner, D.J. (1986). Use of DNA relatedness to study taxonomic relationships among *Clostridium botulinum* type G and similar clostridial species (Abstr). Am. Soc. Microbiol., 86th Annu. Meet. Washington, D.C.
- Suen, J.C., Hatheway, C.L., Steigerwalt, A.G., and Brenner, D.J. (1988). Genetic confirmation of identities of neurotoxigenic *Clostridium baratii* and *Clostridium butyricum* implicated as agents of infant botulism. *J. Clin. Microbiol.* **26**, 2191-2192.
- Ten Broeck, C., and Bauer, J.H. (1922). The tetanus bacillus as an intestinal saprophyte in man. *J. Exp. Med.* **36**, 261.
- Tulloch, W.J. (1919-1920). Report of bacteriological investigation of tetanus on behalf of the war office. *J. Hyg.* **18**, 103.
- van Ermengem, E. (1897). Ueber einem neuen anaeroben Bacillus und seine Beziehungen zum Botulismus. *Z. Hyg. Infektionskrankh.* **26**, 1-56. (English Translation) 1979: *Rev. Infect. Dis.* **1**, 701-719.



- Veronesi, R., Bizzini, B., Focaccia, R., Coscina, A.L., Mazza, C.C., Focaccia, M.T., Carraro, F., and Honningman, M.N. (1983). Naturally acquired antibodies to tetanus toxins in humans and animals from the Galapagos Islands. *J. Infect. Dis.* **147**, 308-311.
- Weickert, M.J., Chambliss, G.H., and Sugiyama, H. (1986). Production of toxin by *Clostridium botulinum* type A cured of plasmids. *Appl. Environ. Microbiol.* **51**, 52-56.
- Wells, C.L., and Balish, E. (1983). *Clostridium tetani* growth and toxin production in the intestines of germfree rats. *Infect. Immun.* **41**, 826-828.
- Wilson, G.S., and Miles, A. (1975). "Topley and Wilson's Principles of Bacteriology, Virology and Immunology" pp. 2237-2238. 6th ed., Vol. 2. Williams and Wilkins, Baltimore, Maryland.
- Wu, J.I.J., Riemann, H., and Lee, W.H. (1972). Thermal stability of the deoxyribonucleic acid hybrids between the proteolytic strains of *Clostridium botulinum* and *Clostridium sporogenes*. *Can. J. Microbiol.* **18**, 97-99.

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